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(54) Title: GELATINASE ANTAGONISTS USED FOR TREATING CANCER

(57) Abstract

This invention describes a method of treating diseases in which gelatinase mediated cell migration and invasion is an essential feature of the pathology of the disease. The method comprises administering an effective amount of an antagonist which inhibits gelatinase mediated cell migration and invasion.

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# GELATINASE ANTAGONISTS USED FOR TREATING CANCER

## FIELD OF THE INVENTION

This invention relates to a method of treating diseases in which gelatinase mediated cell migration and invasion is an essential feature of the pathology of the disease comprising administering an effective amount of an antagonist which inhibits gelatinase mediated cell invasion and migration; to antagonists of said gelatinase mediated cell invasion and migration; to a process of making said antagonists; to pharmaceutical, medical, and diagnostic compositions containing said antagonists and to the medical, diagnostic and therapeutic uses thereof.

## BACKGROUND OF THE INVENTION

In normal tissue, cellular connective tissue synthesis is offset by extracellular matrix degradation, the two opposing effects existing in dynamic equilibrium. Degradation of the matrix is brought about by the action of proteases released from resident connective tissue cells and invading inflammatory cells, and is due, in part, to the activity of several matrix metalloproteinases. Normally these catabolic enzymes are tightly regulated, at the level of their synthesis and secretion and also at the level of their extracellular activity, the latter through the action of a specific inhibitor, TIMP (tissue inhibitor of metalloproteinase), which forms inactive complexes with metalloproteinases.

The matrix metalloproteinases form a group of zinc-dependent enzymes which include the collagenases, gelatinases and stromelysins. Between them they are capable of degrading all the proteins of the extracellular matrix and are therefore implicated in numerous physiological and pathological processes which involve tissue turnover. In the case of gelatinase A (E.C.3.4.24.24,72KDa gelatinase; type IV collagenase) there is increasing evidence correlating enzyme expression and activity with tumour cell invasion and metastasis, and we have now identified this enzyme as a target for therapeutic intervention in a number of diseases.

The accelerated, uncontrolled breakdown of connective tissues by matrix metalloproteinase catalysed resorption of the extracellular matrix is a

feature of many pathological conditions, such as inflammation and infiltration of inflammatory cells; the arthritides e.g. rheumatoid arthritis; corneal, epidermal or gastric ulceration; scleroderma; epidermolysis bullosa; periodontal disease, kidney disease, bone disease and tumour metastasis, angiogenesis and invasion.

Metastasis is the process by which tumour cells leave the primary tumour and migrate to other sites in the body where they colonise giving rise to further tumour masses. In order for a tumour cell to metastasise it must at various stages be capable of invading surrounding tissues. The invasion process is believed to comprise three steps (see Liotta, L., Scientific American, Feb. 1992, 34-41). Adhesion of the tumour cell to the basement membrane is followed by the activation of destructive enzymes that cleave or unravel basement membrane molecules beneath the tumour cell which is in turn followed by migration of the entire tumour cell. Highly invasive tendencies have been correlated with high matrix metalloproteinase levels in human cancers. It is believed that the matrix metalloproteinases are involved in the destruction of basement membrane and other components of the extracellular matrix required to allow the tumour to invade.

It can be expected that the pathogenesis of the conditions and diseases mentioned above is likely to be modified in a beneficial manner by the administration of substances which specifically antagonise the gelatinase mediated cell migration and invasion.

## SUMMARY OF THE INVENTION

In a first aspect the invention provides a method of treating diseases in which gelatinase mediated cell migration and invasion is an essential feature of the pathology of the disease comprising administering an effective amount of an antagonist which inhibits said gelatinase mediated cell migration and invasion.

Diseases in which gelatinase mediated cell migration and invasion is an essential feature of the pathology include for example cancer; inflammatory diseases e.g. asthma, and the athritides such as rheumatoid

arthritis; periodontal disease, corneal, epidermal or gastric ulceration; scleroderma; epidermolysis bullosa, kidney disease and bone disease; autoimmune diseases, e.g. allograft rejection, graft versus host disease, eczema, psoriasis, multiple sclerosis and inflammatory bowel disease e.g. ulcerative colitis and Crohn's disease.

The antagonists for use according to the invention may be useful in the treatment of cancer by inhibiting the invasion of tumour cells.

As used herein the term target cells is used to denote those cells which recruit or use gelatinase, especially gelatinase A, to migrate and/or invade such as, for example, metastatic tumour cells and T-cells (Monsky *et al* (1993) Cancer Res. 53, 3159-3164; Emonard (1992) Cancer Res. 52, 5845-5848, and Romanic and Madri (1994) J. Cell Biol. 125, 1165-1178).

In a preferred embodiment of the first aspect the invention provides a method of treating cancer comprising administering an effective amount of an antagonist which inhibits gelatinase mediated cell migration and invasion.

The antagonists for use in the method according to the invention inhibit both pro and active gelatinase mediated cell migration and invasion and preferably inhibit both progelatinase A and active gelatinase A mediated cell migration and invasion.

Gelatinase A is typical of the matrix metalloproteinase family in that it is secreted as a proenzyme which is activated by proteolytic processing to remove an 80 amino acid propeptide from the N-terminus. The N-terminal domain contains the active site of the enzyme and a matrix binding region that is found only in the gelatinases. The C-terminal domain plays an important role in the cell membrane-mediated activation of the enzyme.

Matrix metalloproteinase activity is controlled at several levels including proenzyme activation and regulation by the general proteinase inhibitor $\alpha_2$-macroglobulin and by the specific metalloproteinase inhibitors, TIMP-1 and TIMP-2. TIMP-1 is a glycoprotein with a molecular mass of about 30

KDa whereas TIMP-2 is unglycosylated with a molecular mass of 23KDa and the two proteins share approximately 40% amino acid homology. All activated matrix metalloproteinases are inhibited by both TIMPs with a 1:1 stoichiometry. Additional TIMPS such as TIMP-3 are also reported to exist.

It is possible to antagonise gelatinase mediated invasion and migration in a number of ways. For example, it is possible by analysing the structure of gelatinase A and the functions of the discrete domains to design antagonists which we believe will still have the ability to bind to target cells and/or extracellular matrix and which optionally have altered catalytic activity, preferably reduced catalytic activity, and which inhibit gelatinase mediated cell migration and invasion.

Gelatinase-A has four discrete domains comprising a propeptide, a catalytic domain, a matrix binding domain and a C-terminal domain.

The propeptide is an 80 amino acid N-terminal domain that maintains the latency of the proenzyme. Activation of gelatinase follows removal of this domain by autolysis.

The catalytic domain of all the MMPs contains a short sequence of identity with thermolysin. In thermolysin, the sequence HELTH is known to contain two of the three histidine residues that co-ordinate the zinc as well as the catalytic glutamic acid. In the absence of structural information the precise $Zn^{2+}$ ligand of gelatinase is not known, but it seems likely that the histidine residues of the conserved HEFGH motif are involved.

We have made use of this sequence information to make an inactive form of gelatinase. By altering the glutamic acid residue at position 375 in the sequence of the molecule we have been able to produce catalytically inactive gelatinase or gelatinase showing reduced catalytic activity. If completely catalytically inactive gelatinase is required, the glutamic acid is preferably replaced by any residue which does not carry the same charge as the native glutamic acid i.e. which is not negatively charged and is most preferably replaced by a glutamine or an alanine residue. It will be

understood by those skilled in the art that a number of amino acid substitutions are possible. It is desirable to choose a substitution which does not interfere with or prevent correct assembly and folding of the gelatinase.

In a second aspect the invention therefore provides a gelatinase antagonist which has altered catalytic activity with respect to wild type gelatinase and which inhibits gelatinase mediated cell migration and invasion with the proviso that said antagonist is not the isolated natural C-terminal region of gelatinase A.

The invention however does extend to the C-terminal domain of gelatinase A when in combination with another protein or non-protein moiety such as for example; one or more domains of gelatinase and/or an antibody or fragment thereof and/or a drug or label. It further extends to the C-terminal domain of gelatinase A in which amino acids have been deleted, added, replaced or modified. The invention also covers a therapeutic, diagnostic, imaging and pharmaceutical composition containing the C-terminal domain of gelatinase A and the uses thereof. This is described more fully herein.

The antagonists according to the second aspect of the invention preferably have reduced or no catalytic activity and are non-naturally occurring antagonists. They are preferably antagonists containing all or a part of gelatinase i.e. one or more gelatinase domains which may optionally be linked to another protein moiety, label and/or drug as will be described more fully herein.

The C-terminal region of wild type gelatinase is believed to be responsible for binding the enzyme to cells expressing a gelatinase receptor or binding site. It is therefore essential that where the C-terminal region is present in the above gelatinase antagonists which are structurally based on gelatinase it is folded appropriately to allow binding of the inhibitors to target cells.

The above gelatinase antagonists are preferably full length gelatinase derivatives i.e. are preferably derivatives of mature secreted progelatinase A as defined by Collier et al (1988) (J. Biol. Chem. 263, 6579-6587). The numbering used herein is where amino acid 1 is the start of the secreted proenzyme as defined by Collier. As used herein the term matrix binding domain denotes amino acids 191-364 where amino acid 1 is the start of the secreted proenzyme.

In a preferred embodiment of this aspect the invention provides a full length gelatinase antagonist in which the glutamic acid residue at position 375 has been deleted or modified or replaced by another amino acid residue or a derivative thereof.

In a particularly preferred embodiment of this aspect of the invention the glutamic acid residue at position 375 is replaced by a glutamine, alanine or aspartic acid residue.

It will be understood however that the amino acid residue could be replaced by another amino acid or derivative with an inappropriate functionality on its side chain and/or by changing the charge and/or polarity of the side chain.

Gelatinase antagonists according to the invention with altered catalytic activity compared to wild type gelatinase could also be produced by adding one or more amino acids to the active site region of the molecule.

We have found that, when activated, antagonists where the glutamic acid residue is replaced by a glutamine or alanine residue have approximately 0.01% of the gelatin degrading activity of wild type gelatinase A.

We have also developed antagonists that show an intermediate activity for example, by replacing the glutamic acid with aspartic acid we have produced an antagonist that, when activated, has approximately 1% of the gelatin degrading activity of wild type gelatinase A.

The gelatinase antagonists according to the invention showing reduced catalytic activity should have an enzyme turnover rate of not greater than about 10% of the wild type activity.

Another method of producing a gelatinase antagonist according to the invention may, for example, be to introduce further changes to the wild type gelatinase or to the gelatinase enzyme with altered catalytic activity such that the propeptide can no longer be removed. Such changes may be made for example by amino acid deletion, addition, replacement, or modification or by chemically modifying the gelatinase.

The gelatinase antagonists according to the invention have many potential therapeutic and diagnostic applications.

As discussed above gelatinase has been implicated in the migration and invasiveness of cells. For example, it has been proposed that certain cells such as tumour cells, have a high level of gelatinase on their surface which is believed to correlate with the ability of the cell to invade and migrate. By administering the gelatinase antagonist we believe it may be possible to displace the wild type enzyme from target cells such as the tumours thereby preventing invasion and migration. The gelatinase antagonist may bind to a binding site or receptor on the cell surface and/or extracellular matrix associated with cells such as tumour cells and anchor the enzyme to the matrix but because the antagonist has reduced or no catalytic activity it can no longer degrade the matrix. By occupying the binding site or receptor sites, the gelatinase antagonist blocks binding of the wild type gelatinase.

The matrix metalloproteinases are secreted as latent proenzymes which are activated to produce active enzyme. We believe that this activation process is mediated through binding/interaction of the gelatinase with 'activator cells' as described by Murphy *et al* (1992) [Biochem J. 283, p.637-641]. Examples of activator cells include fibroblast cells, e.g. human foreskin fibroblast cells. By administering a gelatinase antagonist it may be possible to antagonise the binding of wild type gelatinase to the activator cells thereby inhibiting production of active enzyme. The

gelatinase antagonists may therefore act as an antagonist of wild type gelatinase activation.

As used herein the term gelatinase antagonist is used to denote an antagonist which inhibits gelatinase mediated cell migration and invasion via a mechanism which does not involve interaction with the active site of wild type gelatinase and does not cover those inhibitors of gelatinase which are active site inhibitors which inhibit via an interaction with the active site.

It may be possible to produce other variants which act as antagonists of gelatinase perhaps via a mechanism which involves competing with wild type gelatinase for a binding site or receptor on the cell surface of target cells or extracellular matrix. As discussed previously the C-terminal region of gelatinase is believed to be responsible for binding the enzyme to cells expressing a gelatinase receptor or binding site. The isolated C-terminal domain may therefore be an effective antagonist of the binding of the enzyme gelatinase to target cells. The invention therefore extends to the use of the isolated C-terminal region of gelatinase A in the treatment of diseases in which gelatinase mediated cell migration and invasion is an essential feature of the pathology.

The binding of TIMP.2 to the C-terminal domain may alter the biodistribution of a gelatinase antagonist and may influence the binding of the antagonist to cells and/or matrix molecules and also limit the ability of the antagonist to reduce gelatinase mediated cell migration and invasion. An improvement in the properties of the gelatinase antagonists according to the invention may therefore occur if the TIMP.2 binding properties of the C-terminal domain can be removed. The invention therefore covers also a gelatinase antagonist where the ability of the C-terminal domain component of said gelatinase antagonist to bind TIMP.2 has been substantially reduced. The TIMP.2 binding properties will preferably have been eliminated.

Peptides derived from full length gelatinase enzyme may be made and tested for their cell and/or matrix binding properties in competition

experiments with the enzyme. Those which show the required activity may also be used in the treatment of diseases in which gelatinase mediated cell migration and invasion is an essential feature of the pathology by virtue of their ability to inhibit binding of gelatinase to target cells and/or extracellular matrix.

Following the same principle, it is possible to isolate the matrix-binding region and similarly the matrix binding domain of gelatinase and to use this as an antagonist of gelatinase thereby preventing gelatinase mediated cell migration and invasion perhaps by inhibiting binding of the full length enzyme to the target cell and/or extracellular matrix associated with the target cell. It will be appreciated that the gelatinase used may be either wild-type or gelatinase having altered catalytic activity.

The gelatinase antagonist will preferably be administered at a level in excess of the serum concentration of uncomplexed wild type gelatinase, e.g. at a concentration of greater than 5ng/ml. It will be appreciated that the precise dose of gelatinase antagonist will depend upon the route of administration, the potency of the gelatinase antagonist, and the body weight and pathology of the patient.

The gelatinase antagonist may also usefully be labelled with any suitable detectable label and used as an imaging reagent to detect those cells or extracellular matrix expressing a gelatinase receptor or matrix or cell specific binding site with an affinity for gelatinase. In general, the gelatinase antagonist will be labelled with an atom, group or other molecule. The label may be for example a radiolabel such as $^{125}I$, $^{131}I$, $^{32}P$, $^{35}S$, $^{112}In$, $^{99M}Tc$, $^{14}C$ or $^{3}H$, a chromophore or fluorophore such as fluorescein or rhodamine, or an atom or group capable of being monitored by NMR or ESR spectroscopy, or an enzyme, substrate, inhibitor or cofactor for a chemical reaction, a product or a substrate of which is itself detectable, for example by colorimetric, fluorometric or chemiluminometric means. The gelatinase antagonist may also be used as a means of delivering a drug to a target site.

The term drug as used herein is intended to mean any physiologically active substance, antibacterial, antiviral or antifungal compound. Particular physiologically active substances include antineoplastic agents, including cytotoxic and cytostatic agents, hormones, anti-inflammatory compounds and substances active as cardiovascular e.g. fibrinolytic and central nervous system agents; toxic radioisotopes, heavy metals, enzymes and complement activators.

Particular antineoplastic agents include cytotoxic and cytostatic agents, for example alkylating agents, such as nitrogen mustards (e.g. L-chlorambucil, melphalan, mechlorethamine, cyclophosphoramide or uracil mustard) and derivatives thereof, triethylenephosphoramide, triethylenethiophosphoramide, busulphan or cisplatin; antimetabolites such as methotrexate, fluorouracil and floxuridine, cytarabine, mercaptopurine, thioguanine, fluoroacetic acid or fluorocitric acid; antibiotics, such as bleomycins, doxorubicin, daunorubicin, mitomycins, actinomycins, plicamycin, calicheamicin or esperamicin; mitosis inhibitors, such as etoposide, vincristine or vinblastine and derivatives thereof; aklaloids such as ellipticine; polyols such as taxicin-I or taxicin-II; hormones such as androgens (e.g. dromostanolone or testolactone), progestins (e.g. megestrol acetate or medroxyprogesterone acetate), estrogens (e.g. diethylstilbestrol diphosphate, polyestradiol phosphate or estramustine phosphate) or antiestrogens (e.g. tamoxifen); ureas such as hydroxyurea; hydrazines, such as procarbazine; or imidazoles such as dacarbazine.

The gelatinase antagonist may also be linked to another protein moiety such as for example an antibody. This conjugate should preferably have an increased half-life compared to unconjugated gelatinase. The antibody may be a non-specific antibody, i.e. a non gelatinase specific antibody. A mixture of antibodies with varying specificities may be used. The antibody or gelatinase may be labelled with any suitable detectable label.

As used herein the term antibody covers also antibody fragments. The antibody or antibody fragment may in general belong to any immunoglobulin class. Thus, for example, it may be an immunoglobulin M antibody or, in particular an immunoglobulin G antibody. The antibody or

fragment may be of animal, for example mammalian origin and may be for example of murine, rat or human origin. It may be a natural antibody or a fragment thereof, or, if desired, a recombinant antibody or antibody fragment, i.e. an antibody or antibody fragment which has been produced using recombinant DNA techniques.

Particular recombinant antibodies or antibody fragments include those having an antigen binding site at least part of which is derived from a different antibody, for example those in which the hypervariable or complementarity determining regions of one antibody have been grafted into the variable regions of a second different antibody such as chimeric and CDR-grafted antibodies (see for example European Patent Specification No. 239400 and International Patent Application No. WO 91/09967).

The antibody or antibody fragment is preferably of monoclonal origin.

Antibody fragments include for example fragments derived by proteolytic cleavage of a whole antibody e.g. $F(ab')_2$, Fab' or Fab fragments.

Therapeutically it may be necessary to maintain high levels of the gelatinase antagonist within the tissues for relatively long periods. This may be achieved for example by fusing gelatinase antagonist effector regions, e.g. all or part of the gelatinase A C-terminal domain and all or part of the gelatinase matrix binding domain; to another molecule such as an antibody or fragment thereof which has properties that improve the pharmacokinetics of the chimeric molecule and the invention extends also to this type of gelatinase antagonist.

Gelatinase antagonist effector functions may be combined with an antibody or fragments thereof, by replacing one or more of the antibody CDRs with the effector region of the gelatinase antagonist, and the invention extends also to this type of gelatinase antagonist.

The antibody or drug or label may be attached directly or indirectly through a linker group to the gelatinase antagonist. Direct linkage is to be

understood to mean peptide bond formation and indirect linkage is to be understood to mean linkage via a synthetic bridging group.

In a fourth aspect the invention provides a method of delivering a drug or antibody to a target site characterised in that said drug or antibody is directly or indirectly linked to a gelatinase antagonist, said antagonist inhibiting gelatinase mediated cell migration and invasion

In a fifth aspect the invention provides a therapeutic, imaging or diagnostic composition comprising a gelatinase antagonist and a pharmaceutically acceptable excipient said antagonist inhibiting gelatinase mediated cell migration and invasion.

In a further aspect the invention provides a process for the production of a pharmaceutical composition comprising bringing a gelatinase antagonist which inhibits gelatinase mediated cell migration and invasion into association with a pharmaceutically acceptable carrier, excipient or diluent.

The therapeutic, imaging or diagnostic composition may comprise gelatinase antagonists which may be labelled or coupled to an antibody or drug molecule.

The invention also extends to a method of therapy, imaging or diagnosis comprising administering an effective amount of an antagonist of gelatinase which inhibits gelatinase mediated cell migration and invasion.

Pharmaceutical, therapeutic, imaging and diagnostic compositions for use according to the present invention may be formulated in conventional manner, optionally with one or more physiologically acceptable carriers, diluents or excipients.

Antagonists for use according to the present invention may be formulated for oral, buccal, parenteral or rectal administration or in a form suitable for nasal administration or administration by inhalation or insufflation.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g. pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g. lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g. potato starch or sodium glycollate); or wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents; emulsifying agents; non-aqueous vehicles; and preservatives. The preparations may also contain buffer salts, flavouring, colouring and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated to give controlled release of the gelatinase antagonist For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

The gelatinase antagonist may be formulated for local administration into a desired area such as a joint or for parental administration by injection e.g. by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use.

The gelatinase antagonist may also be formulated in rectal compositions such as suppositories or retention enemas, e.g. containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the gelatinase antagonist may also be formulated as a depot preparation. Such long

acting formulations may be administered by implantation or by intramuscular injection.

For nasal administration or administration by inhalation the gelatinase antagonist for use according to the present invention is conveniently delivered in the form of an aerosol spray presentation for pressurised packs or a nebuliser, with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas.

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the inhibitor gelatinase. The pack or dispenser device may be accompanied by instructions for administration.

The gelatinase antagonists may be prepared using methods well known in the art, and in the accompanying examples. For example, they may be produced by recombinant DNA technology via expression of a truncated gene(s) or they may be produced by conventional amino acid synthetic techniques, or they may be produced by appropriate purification of fragments generated by chemical or enzymatic cleavage of the gelatinase molecule.

Where the gelatinase antagonist which has reduced catalytic activity is being produced via a recombinant DNA approach, it will be readily apparent to the man skilled in the art that the naturally occurring glutamic acid residue at position 375 should be replaced by a naturally occurring amino acid.

An alternative amino acid may be introduced at position 375 by for example site directed mutagenesis, or by for example using PCR amplification followed by appropriate restriction enzyme digestion of the amplified product and ligation with a nucleotide containing the desired mutated sequence coding for the required inhibitor gelatinase.

If the gelatinase antagonist is produced by chemical synthesis the glutamic acid residue at position 375 may be chemically modified to produce catalytically inactive gelatinase. It may for example be alkylated. Other methods of chemically or otherwise modifying amino acid residues are well known in the art.

Another method of producing a gelatinase antagonist according to the invention may, for example, be to chemically reduce the gelatinase provided that this does not interfere with either the C-terminal region binding to the target cell or the matrix binding region.

An assay for the identification of antgonists which disrupt the binding of gelatinase to target cells and/or extracellular matrix may be developed using standard techniques such as binding assays and FACS based assay systems.

Gelatinase antagonists according to the invention may be identified by testing in assays of cell invasion and migration such as by measuring inhibition of lung nodule formation described in the accompanying examples; matrigel invasion models; inhibition of metastatic spread of A375 cells to bone (Arguello *et al* (1988) (Cancer Res. 48 6876-6881 and Nakai *et al* (1992) Cancer Res. 52 5395-99)); an experimental allergic encephalomyelitis model (Martenson) (1984) in Experimental Allergic Encephalomyelitis: A useful Model for Multiple Sclerosis ed. E.C. Alvord Jr. pp 146-511, New York: Liss.) in an experimental arthritis model (Follard and Terlain (1988) Agents and Actions 25 1/2; Lewthwaite *et al* (1994) The J. of Rheumatology 21 3, 467-472); experimental atherosclerosis model (Bennett *et al* (1994) J. Clin. Invest. 93 820-828) or in an Experimental Ulcerative Colitis and Crohn's Disease model (Lionetti *et al* (1993) Gastroenterology 105 373-381).

The invention is illustrated with reference to the following non-limiting examples and with reference to the following figures in which:

## BRIEF DESCRIPTION OF THE FIGURES

**FIGURE 1.**

**Construction of complete human gelatinase-A coding sequence.**

A: Schematic representation of the steps taken to generate a complete coding sequence.

B: The oligonucleotide generated sequence used to rebuild the cDNA truncated at the 5' NarI site. Amino acids are shown in a single letter code and are numbered from the first residue of the secreted proenzyme.

**FIGURE 2.**

**Construction of GL-A($E_{375}Q$), and GL-A($E_{375}D$) and GL-A($E_{-375}A$) coding sequences.**

A: Schematic representation of the steps taken to generate the mutant enzyme sequences.

B: The oligonucleotides used to mutate the wild type gelatinase-A DNA sequence to GL-A($E_{375}Q$), GL-A($E_{375}A$) andGL-A($E_{375}D$).

**FIGURE 3.**

**SDS-polyacrylamide gel electrophoresis of the purified recombinant progelatinase A mutants.**

Lane 1, marker proteins; lane 2, wild type progelatinase A; lane 3, proE$^{375\rightarrow}$A; lane 4, proE$^{375\rightarrow}$D; lane 5, pro E$^{375\rightarrow}$Q.

**FIGURE 4.**

**Trypsin digestion of wild type progelatinase A and proE$^{375\rightarrow}$Q.**

(A) Wild type progelatinase A and (B) proE$^{375\rightarrow}$Q at a concentration of 2μM were incubated with bovine trypsin (0.4μM) at 23°C in either the absence (lanes 2 to 6) or presence (lanes 7 to 11) of 28mM EDTA. At the time points indicated, aliquots from each incubation were removed and the trypsin inactivated with 4mM phenylmethanesulfonyl fluoride before their analysis by SDS-polyacrylamide gel electrophoresis. Lane 1, marker proteins; lanes 2 and 7, Oh; lanes 3 and 8 2h; lanes 4 and 9, 8h; lanes 5 and 10, 24h. The samples run in lanes 6 and 11 are the 24h time points taken from separate incubations performed in the absence of trypsin.

**FIGURE 5.**

**Formation of TIMP-2/progelatinase A complexes.**

Increasing concentrations of wild type or mutant forms of progelatinase A were added to a fixed concentration of TIMP-2 and the remaining free TIMP-2 concentration measured by ELISA. Results are presented as a percentage of the signal obtained in the absence of any progelatinase A.

**FIGURE 6.**

**Activation of wild type progelatinase A and proE$^{375\rightarrow D}$.**

(A) Wild type progelatinase A (O) and proE$^{375\rightarrow D}$ (●) were incubated at a concentration of 2.7μM in the presence of 1mM APMA at 23°C. At the indicated time points, aliquots were removed and assayed for activity using the synthetic substrate McaPLGLDpaAR. Results are presented as a percentage of the two maximum activities obtained. (B) Samples taken during the APMA activation of proE$^{375\rightarrow D}$ were analysed by SDS-polyacrylamide gel electrophoresis. Lane 1, marker proteins; lane 2, Oh; lane 3, 1h; lane 4, 2h; lane 5, 4h; lane 6, 8h; lane 7, 24h; lane 8, 48h; lane 9, 48h no APMA. Lanes 10 and 11 are the 0h and 48h samples taken from the proE$^{375\rightarrow D}$ activation analysed by gelatin zymography. 100ng of mutant were loaded on both lanes. The positions of migration of wild type pro and active gelatinase A are as indicated.

**FIGURE 7.**

**Analysis by SDS-polyacrylamide gel electrophoresis of the activation of proE$^{375\rightarrow A}$ and proE$^{375\rightarrow Q}$ by APMA or Δ418-631 gelatinase A.**

(A) ProE$^{375\rightarrow A}$ (lanes 2 to 6) and pro E$^{375\rightarrow Q}$ (lanes 7-11) at a concentration of 7.1μM were incubated at 37°C in the presence of 1mM APMA. Aliquots from each incubation were removed at the indicated time points. Lane 1, marker proteins; lanes 2 and 7, Oh; lanes 3 and 8, 24h; lanes 4 and 9, 96h; lanes 5 and 10 168h. The samples run in lanes 6 and 11 are the 168h time points taken from separate incubations performed in the absence of APMA. (B) ProE$^{375\rightarrow A}$ (lanes 2 to 6) and proE$^{375\rightarrow Q}$ (lanes 7 to 11) were incubated at 37°C for the times indicated in either the presence of absence of Δ418-631 gelatinase A and then purified as described in Experimental Procedures. Lane 1, marker proteins; lanes 2 and 7, Oh; lanes 3 and 8, 16h; lanes 4 and 9, Oh plus Δ418-631 gelatinase A after heparin Sepharose CL-6B chromatography; lanes 5 and 10, 16h plus Δ418-631 gelatinase A; lanes 6 and 11, 16h plus Δ418-631

gelatinase A after heparin Sepharose CL-6B chromatography, lane 12, Δ418-631 gelatinase A. (C) Gelatin zymogram of wild type and mutant gelatinase A samples. Lane 1, 30pg wild type progelatinase A; lane 2, 30pg wild type gelatinase A activated by incubation with 1mM APMA for 24h at 23°C; lane 3, 30pg APMA-activated Δ418-631 gelatinase A; lane 4, 100ng $proE^{375 \rightarrow A}$; lane 5, 1μg $proE^{375 \rightarrow A}$ incubated with 1mM APMA for 196h at 37°C; lane 6, 300ng $proE^{375 \rightarrow A}$ incubated with Δ418-631 gelatinase A for 16h at 37°C and purified by heparin Sepharose CL-6B; lane 7, 100ng $proE^{375 \rightarrow Q}$; lane 8, 1μg $proE^{375 \rightarrow Q}$ incubated with 1mM APMA for 196h at 37°C; lane 9, 300ng $proE^{375 \rightarrow Q}$ incubated with Δ418-631 gelatinase A for 16h at 37°C and purified by heparin Sepharose CL-6B.

**FIGURE 8.**

**Effect of gelatinase antagonists on matrigel invasion and lung nodule formation.**

(a) Matrigel invasion by C127 cells and C127 (.02/3) cells expressing gelatinase-A ($GL\text{-}A_1$) in the absence, (-); or presence of 25 μg per well of the gelatinase antagonist ($E_{375}>Q$). The p values were calculated by comparing the number of cells in the lower chamber after incubation in the presence or absence of the gelatinase antagonist.

(b) Matrigel invasion by C127 cells or C127 (.02/3) cells expressing gelatinase-A ($GL\text{-}A_1$) or two independently isolated C127 cell lines secreting the gelatinase antagonist ($E_{375}>Q_1$ and $E_{375}>Q_2$). The p values were calculated by comparing the number of gelatinase antagonist secreting cells in the lower chamber with the number of parental C127 cells.

(c) Lung nodule formation after tail vein injection of Balb/c nu.nu mice with the .02/3 gelatinase-A secreting cell ($GL\text{-}A_1$) after it had been incubated in the absence (-); or in the presence of either 25 μg wild type (Wt. GL-A) or the gelatinase antagonist ($E_{375}>A$).

(d) As for (c) except the effect of preincubating or co-injecting the gelatinase antagonist was compared.

## DETAILED DESCRIPTION OF THE SPECIFIC EMBODIMENTS OF THE INVENTION

### EXAMPLE 1

A) Cloning and Expression of Recombinant Human Gelatinase-A --- Oligonucleotides based on the published sequence of human gelatinase A (Collier *et al.*, 1988) were used to identify corresponding sequences within a mixed human tumour library that was kindly provided by Dr R Breathnach (Muller *et al.*, 1988). The longest sequence was found by sequencing (Sanger *et al.*, 1977) to be identical with that described by Collier et al., (1988) except it lacked approximately 100 nucleotides from the 5' end of the coding sequence as shown schematically in Fig. 1, a. The missing nucleotides were generated from a series of overlapping oligonucleotides that were based on the 5' coding sequence of the human gelatinase-A gene (Huhtala *et al.*, 1990) with changes in the third nucleotide of several codons being incorporated in order to reduce the G/C content of this section of DNA whilst maintaining the protein sequence as shown in Fig. 1, b. A 5' Hind III site and a Kozak consensus sequence (Kozak, 1987) were included upstream of the ATG initiating codon to facilitate cloning and to optimise translational efficiency. The oligonucleotide generated sequence was ligated to the cDNA at a convenient NarI site located close to the 5' end of the truncated cDNA. This NarI site is not present in the sequence reported by Collier *et al.*, (1988) and was not reformed as a result of the ligation.

Much of the approximately 800 bp 3' untranslated region of the gelatinase-A cDNA was removed by digestion with Nar I as shown in Fig. 1,a. Subsequent treatment with the Klenow enzyme allowed EcoRI linkers to be ligated to the blunt ended cDNA. The 5' and 3' modified gelatinase-A coding sequences were reassembled through a common BamHI site and resequenced to confirm the changes before being inserted between the unique Hind III and EcoRI sites of the mammalian cell expression vector pEE12 (Murphy et al., 1991) to generate pEE12-Gl-A.

Two to three weeks after electroporation of pEE12-GL-A into NSO mouse myeloma cells, colonies were identified and screened for secretion of gelatinase A by analysis of conditioned medium on substrate gels and inactivity assays as previously described (Murphy et al., 1992). Selected colonies were expanded, cloned and adapted for suspension growth in serum-free medium via Erlmeyer flasks and roller bottles to generate conditioned medium for enzyme purification.

B) Cloning and expression of gelatinase-A mutants: GL-A($E_{375}Q$) and GL-A($E_{375}A$) ---

pEE12-GL-A was subjected to 20 rounds PCR amplification with Taq 1 polymerase (Mullis and Faloona, 1987). This was undertaken in the presence of an oligonucleotide that primes downstream from the Kpn 1 site shown in Fig. 2A (primer 1) and either mutagenic oligonucleotide 1: to make GL-A($E_{375}Q$); or mutagenic oligonucleotide 2: to make GL-A($E_{375}A$). The oligonucleotide sequences are shown in Fig. 2B. The amplified product was digested with EcoRI and KpnI to generate a 480 bp DNA fragment that was ligated into a recipient vector that contained the wild type gelatinase-A sequence that had also been digested with EcoRI and Kpn1. By this means the wild type gelatinase-A sequence was converted to either the GL-A($E_{375}Q$) or GL-A($E_{375}A$) mutant sequence (Fig. 2A). The DNA sequences of the two mutants were confirmed by the Sanger method (1977) using a series of overlapping oligonucleotides. The full length mutant sequences were subcloned into the pEE12 mammalian cell expression vector and transfected into NSO cells which were used to generate mutant enzyme containing conditioned media as decribed above for the wild type enzyme.

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**EXAMPLE 2**

**Purification of the Mutant 72kDa Progelatinases.**

Conditioned medium from the NSO cell lines was incubated with gelatin Sepharose 4B (Pharmacia Ltd.) at 4°C for 16h (approx. 25ml gelatin Sepharose/litre supernatant). The gelatin Sepharose was then collected by vacuum filtration, resuspended in 25 mM Tris/HCl, 10mM $CaCl_2$, 30mM NaCl, pH 7.5 and poured into a column ($2cm^2$x20cm). After washing with 500ml of 25mM Tris/HCl, 10mM $CaCl_2$, 1M NaCl, pH 7.5 protein bound to the gelatin Sepharose was eluted with wash buffer + 10% DMSO. Analysis by SDS-polyacrylamide gel electrophoresis established that all three mutant forms were purified using this one-step protocol. The purified mutants were then desalted into 25 mM Tris/HCl, 10mM $CaCl_2$, 30mM

NaCl, pH 7.5 by gel filtration using Sephadex G-25 and aliquots stored at -70°C. The solutions were analysed by SDS-polyacrylamide gel electrophoresis and gelatin zymography to establish the purity of the progelatinase A mutants. This revealed that proE$^{375}\rightarrow$A and proE$^{375}\rightarrow$Q were purified by the above method but that the proE$^{375}\rightarrow$D sample contained a low level of contaminating progelatinase B. This was removed by passing the solution through a column of Con A Sepharose that binds only the glycosylated progelatinase B (Wilhelm *et al* J. Biol. Chem. 264 17213-17211 (1989). The concentration of the purified progelatinase A mutants was determined by absorbance at $A_{280}$ using $\varepsilon$ = 122800 $M^{-1}cm^{-1}$ (Crabbe *et al* (1993) Eur. J. Biochem, 218, 431-438).

**Gel Electrophoresis**

SDS-PAGE was performed using precast 10-20% acrylamide gradient gels (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) samples to be analysed typically contained 1µg of protein and were prepared by boiling in standard running buffer (Laemmli UK (1970) Nature 227 680-685) which included 2% (v/v) β-mercaptoethanol and 30mM EDTA. Completed gels were stained for protein using Coomassie Blue R-250 and quantitated as required using a computing Densitometer Model 300A (Molecular Dynamics, Sunnyvale, CA). Gelatin zymography was performed using 10% SDS-polyacrylamide gels containing 0.6mg/ml gelatin as previously described (Hibbs *et al*, (1985) J. Biol. Chem. 260 2493-2500; Crabbe *et al* (1993) Eur. J. Biochem, 218, 431-438).

**Progelatinase A activation**

Wild type progelatinase A and the three mutants were all activated by incubation with 1mM 4-(aminophenyl)mercuric acetate (APMA) at either 23°C or 37°C as indicated. ProE$^{375}\rightarrow$A and proE$^{375}\rightarrow$Q were also activated by Δ418-631 gelatinase A, a deletion mutant of the wild-type enzyme that lacks the C-terminal domain but has full catalytic activity (Murphy *et al*, Biochem J. 283 637-641 (1992)). Δ418-631Progelatinase A was first activated by its incubation with 1mM APMA for 4h at 23°C and the APMA then removed by gel filtration into buffer A + 30mM NaCl. 5.5µM solutions of proE$^{375}\rightarrow$A and proE$^{375}\rightarrow$Q were incubated at 37°C for

16h in the presence of 0.9μM active Δ418-631 gelatinase A (as determined by TIMP-2 titration) and the mixture then applied to a heparin Sepharose CL-6B column. After washing the column with buffer A + 30mM NaCl, the bound proteins, which included activated $E^{375 \rightarrow A}$ or $E^{375 \rightarrow Q}$, were eluted using buffer A+ 0.6M NaCl. Δ418-631 Gelatinase A fails to bind to heparin Sepharose at pH7.5 because it lacks the C-terminal domain (Crabbe *et al.*, J. Eur. J. Biochem 218, 431-438 (1993)).

**Enzyme Assay**

Gelatinase A activity was routinely assayed by following the increase in fluorescence that accompanied hydrolysis of the synthetic substrate, McaPLGLDpaAR, as previously described (Knight *et al.*, FEBS Lett. 296 263-66 (1992), Willenbrock *et al.*, Biochemistry 32, 4330-37 (1993)). The concentration of active $E^{375 \rightarrow D}$ was determined by titration against TIMP-1 and $k_{cat}/K_m$ measured at a substrate concentration of 0.5μM (i.e. [S] $\ll K_m$). Activity against gelatin was assayed using $^{14}C$-labelled denatured rat type I collagen as previously described (Murphy *et al.*, Biochem J. 199 807-811 (1981)) except that the reaction was performed at 23°C.

**Kinetic Analysis of Data**

The value of the rate constant for the association of the active $E^{375 \rightarrow D}$/TIMP-1 inhibitory complex ($k_{on}$) was determined by addition of TIMP-1 (1-2nM) to a reaction mixture containing active enzyme (0.5nM) and substrate (0.5μM). The reaction was followed continuously until a steady-state velocity was reached and progress curves were analysed by using the Enzfitter program (Leatherbarrow, (1987) Enzfitter software, Elsevier Biosoft, Cambridge, UK.) and the following equation:

$$P = v_s t + [v_o - v_s)\ (1-\gamma) / \lambda\gamma]\ \ln[(1 - \gamma e^{\lambda\tau}) / 1 - \gamma)] \qquad (1)$$

in which P is the product concentration, $v_o$ and $v_s$ are the initial and steady-state velocities respectively and $\gamma$ and $\lambda$ are parameters described previously (Willenbrock *et al.*, Biochemistry 32 4330-4337 (1993)). The value of $\kappa_{on}$ was calculated using the equation.

$$\kappa_{on} = \lambda / [(Et + It)^2 - 4E_tI_t]^{1/2} \qquad (2)$$

in which $E_t$ and $I_t$ are the total enzyme and inhibitor concentrations.

**TIMP-2 Binding**

The ability of the three mutants of progelatinase A to bind TIMP-2 was determined by an enzyme-linked immunoabsorbant assay using an antibody that preferentially recognises uncomplexed TIMP-2 over TIMP-2 bound to progelatinase A. Increasing concentrations of the mutants were assessed for their ability to decrease the uncomplexed TIMP-2 concentration. Similar assays were also performed on wild-type progelatinase A and Δ418-631 progelatinase A.

## RESULTS

**Purification and Analysis of the progelatinase A Mutants**

All three progelatinase A mutants were purified to >95% homogeneity as determined by SDS-polyacrylamide gel electrophoresis where they comigrated with wild type progelatinase A at the expected $M_r$ of 72000 (Figure 3). The N-terminal amino acid sequence of proE$^{375\rightarrow A}$ was found to be APSPIIKFPG, which is identical to that of naturally secreted progelatinase A (Collier *et al.*, J. Biol. Chem. 263 6579-6587 (1988)). The effect of E$^{375}$ alteration upon the overall conformation of progelatinase A was adjudged to be minimal because the relative molecular masses and rates of appearance of the fragments of proE$^{375\rightarrow Q}$ generated by trypsin digestion were similar to those of the wild type proenzyme (Figure 4). The addition of EDTA accelerated trypsin digestion indicating that, like the wild type enzyme, the mutant utilises metal cations ($Ca^{2+}$ and $Zn^{2+}$) in order to adopt its correct conformation. ProE$^{375\rightarrow A}$ and proE$^{375\rightarrow D}$ gave similar patterns of trypsin digestion (results not shown). The three progelatinase A mutants were also able to form complexes with TIMP-2 to the same extent as the latent wild type enzyme. This was shown by incubating TIMP-2 with increasing concentrations of progelatinase A and then measuring the decrease in the free TIMP-2 concentration by ELISA (Figure 5). Complex formation is reliant upon the C-terminal domain of gelatinase A as demonstrated by the inability of Δ418-631 progelatinase A to elicit any decrease in the TIMP-2 signal.

**Activation of the Progelatinase A Mutants**

ProE$^{375\rightarrow D}$ was activated by APMA, though at a reduced rate in comparison to the wild type proenzyme (Figure 6). Maximum activity was obtained after 24h at 23°C but analysis by SDS-polyacrylamide gel electrophoresis revealed that at this time point not all of the $M_r$ 72000 proE$^{375\rightarrow D}$ had been converted to the $M_r$ 66000 E$^{375\rightarrow D}$; the only other active species on the gelatin zymogram. Upon storage the activated mutant proceeded to lose its activity at a greater rate than the wild type enzyme (results not shown). TIMP-1 was titrated against the E$^{375\rightarrow D}$ activity obtained after 48h of incubation and extrapolation of the straight line obtained when residual activity was plotted against TIMP-1 concentration (correlation coefficient = 0.99) gave an ordinate intercept of 1.3μM. Densitometric gel scanning revealed that at 48h E$^{375\rightarrow D}$ constituted approximately 60% of the total protein (i.e. 1.6μM) giving a ratio of TIMP-1: E$^{375\rightarrow D}$ that results in complete inhibition of 0.8:1. The most likely stoichiometry for the interaction is therefore 1:1. Using 1.3μM as the active enzyme concentration gave a $\kappa_{cat}/K_m$ value for E$^{375\rightarrow D}$ of $5.7 \times 10^4$ $M^{-1}s^{-1}$, which is ten times lower than that of wild type gelatinase A (Murphy *et al.*, Biochem J. 283, 637-641 (1992)). The specific activity of E$^{375\rightarrow D}$ against $^{14}$C-labelled gelatin was 11μg gelatin degraded $min^{-1}$ $\mu mol^{-1}$ enzyme against a value of 1260$\mu g^{-1}min^{-1}\mu$mol enzyme for wild type gelatinase A.

APMA was only able to convert proE$^{375\rightarrow A}$ and proE$^{375\rightarrow Q}$ into their $M_r$ 66000 forms at 37°C and at an extremely slow rate (Figure 7A). At no stage during this process was any activity against McaPLGLDpaAR detected and, like their parent proenzymes, the $M_r$ 66000 forms displayed no activity on the gelatin zymogram (Figure 7C). We have recently shown that $M_r$ 66000 gelatinase A can activate high concentrations of the proenzyme by autolysis (Crabbe *et al.*, Eur. J. Biochem in press (1993) 218, 431-438). In this study, active Δ418-631 gelatinase A was used to perform the autolysis as it possesses the same specific activity as the full length wild type enzyme (Murphy *et al.*, J. loc. cit (1992)) and can subsequently be removed by heparin Sepharose CL-6B chromatography (Crabbe *et al.*, Eur. J. Biochem. (1993) 218, 431-438). When incubated with active Δ418-631 gelatinase A for 16h at 37°C, approximately half (as deduced by densitometric gel scanning) of the proE$^{375\rightarrow A}$ and proE$^{375\rightarrow Q}$

originally present was converted to their respective $M_r$ 66000 forms (Figure 7B). The N-terminal amino acid sequence of $E^{375}\rightarrow A$ was found to be YNFFP, which is identical to that of APMA-activated wild type gelatinase A and indicates that cleavage at $N^{80}$-$Y^{81}$ had occurred (Stetler-Stevenson *et al.*, J. Biol. Chem. 264 1353-1356 (1989)). The gradual decrease in proenzyme concentration during the incubation led to a concomitant decrease in its rate of conversion while the $M_r$ 66000 form itself became susceptible to autolysis (results not shown) resulting in the formation of the $M_r$ 31000 species previously identified as the gelatinase A C-terminal domain (Crabbe *et al.*, Eur. J. Biochem. (1993) 218, 431-438). 50% conversion to the $M_r$ 66000 form therefore represents the maximum value achievable using this method. Although heparin Sepharose chromatography removed most of the active Δ418-631 gelatinase A, the gelatin zymogram revealed that a small quantity (approximately 5nM) remained, thereby preventing the accurate quantitation of $E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$ activity using the McaPLGLDpaAR assay. The zymogram does reveal, however, that, when this mode of activation is employed, both $E^{375}\rightarrow A$ and $E^{375}\rightarrow Q$ possess a degree of gelatin degrading activity (approximately 0.01% of that displayed by wild type gelatinase A).

**EXAMPLE 3**

**New Gelatinase A Mutants**

1. Matrix Binding Domain Dead Mutant

**Background:** Poor localisation to the cell surface might be caused by the presence of the gelatin binding domain which will locate the mutant to the extracellular matrix instead. Removal of the gelatin binding domain of the $E^{375}$ —>A mutant might, therefore improve its pharmacokinetics.

**Description:** $E^{375}$ —>A mutation + deletion of amino acids 191-364.

**Expression:** For example, as described by Murphy *et al*, J. Biol. Chem., 269: 6632-6636 (1994) except that the matrix binding domain is deleted from gelatinase-A which has the $E^{375}$ —>A substitution (Crabbe *et al.*, Biochemistry, 33: 6684-6690 (1994).

**Purification:** Gelatin Sepharose cannot be used to purify this mutant. Instead it can be purified from the conditioned medium of

NSO cells as follows: Apply to column of S-Sepharose equilibrated with 25mM Tris/HCl, 10mM $CaCl_2$, pH 7.5. Wash off unbound proteins with the same buffer and then elute the bound proteins (which includes the mutant) with equilibration buffer + 100mM NaCl. This fraction should be buffer exchanged to remove the NaCl and then reapplied to S-Sepharose and eluted using a gradient of 0-200mM NaCl.

**Properties:** This mutant will have no detectable activity against gelatin. It will also be unable to bind to gelatin or collagen.

2. Unactivatable Mutants (a), (b) and (c)

**Background:** The $N^{80}$-$Y^{81}$ peptide bond must be cleaved by MMP activity to remove the progelatinase A propeptide and activate the enzyme. Alteration of $Y^{81}$ to an amino acid that is not hydrophobic (eg to a serine) will prevent cleavage of this bond. It might be that clearance of gelatinase A is increased when the propeptide is removed so this mutation may improve the pharmacokinetics. Also, alteration of $Y^{81}$ of the wild-type proenzyme might be another way of making a "dead enzyme" (i.e. it has a functional active site but it will never get activated).

**Description:** a) Alteration of main acid $Y^{81}$ to a non-hydrophobic amino acid or W.

b) $E^{375}$ —>A mutation + alteration of amino acid $Y^{81}$

c) $E^{375}$ —>A mutation + deletion of amino acids 191-364 + alteration of amino acid $Y^{81}$.

**Expression:** a), b) and c) Essentially as described for the $E^{375}$ —>A (Crabbe *et al*, Biochemistry, 33: 6674-6690 (1994) or the matrix binding domain deletant (Murphy *et al*, J. Biol. Chem. 269: 6632-6636, (1994)).

**Purification:** a) and b) As for the $E^{375}$ —>A mutant.

c) As for the Matrix Binding Domain Dead Mutant.

**Properties:** a) full activity against gelatin but the propeptide will not be removed *in vivo*.

b) 0.01% activity against gelatin but the propeptide will not be removed *in vivo*

c) no detectable activity against gelatin and the propeptide will not be removed *in vivo*. It will also be unable to bind to gelatin or collagen.

3. C-terminal Domain

**Background:** This probably represents the simplest cell-binding fragment of gelatinase A.

**Description:** Amino acids 415-631 of gelatinase A.

**Expression:** This will be obtained as a breakdown product of progelatinase A.

**Purification:** Wild-type progelatinase A expressed by NSO cells and purified as previously described (Murphy *et al*., Biochem J. 283, 637-641 (1992)) Prolonged incubation in the presence of 1mM APMA (e.g. 24h at 37°C) will give rise to various breakdown products, one of which will be the gelatinase A C-terminal domain. This can be separated away from the other fragments by treating the sample with gelatin Sepharose 4B, which will bind all the other fragments but will not bind the C-terminal domain. A further purification step would be to apply the non-binding fraction to a column of heparin Sepharose CL 6B equilibrated with 25mM Tris/HCl, 10mM $CaCl_2$, pH 7.5 Bound proteins, which will include the C-terminal domain, can be eluted from the column by a gradient of 0-600mM NaCl. Fractions containing the C-terminal domain will be combined and stored at 4°C.

**Properties:** No catalytic activity. No gelatin or collagen binding activity. Should still bind to cells.

4. TIMP.2 Non-Binding Mutant

**Background:** The binding of TIMP.2 to the C-terminal domain may alter the biodistribution of the gelatinase antagonist and may

influence the binding of the antagonist to cells and or matrix molecules and also limit the ability of the antagonist to reduce gelatinase mediated cell migration and invasion.

Improvement in the properties of the gelatinase antagonist may therefore occur if the TIMP.2 binding properties of the C-terminal domain can be removed.

**Description:** $E^{375}$ —>A mutation, C-terminal domain alone or C-terminal domain - antibody chimera in which the gelatinase-A C-terminal domain has been mutated to eliminate its TIMP.2 binding properties.

**Expression:** Recombinant DNA methodologies such as alanine scanning mutageneses are used to generate alanine residue substitutions for amino acids within the progelatinase-A C-terminal domain. Mutants of progelatinase-A with reduced TIMP.2 binding properties are identified in an ELISA format in which an antibody which recognises the gelatinase is used as a capture reagent to bind the enzyme to a solid phase and a second antibody which recognises TIMP.2 is used to determine whether the captured progelatinase is capable of binding TIMP.2. Mutants exhibiting reduced C-terminal domain TIMP.2 binding are selected for scaled up expression and purification, followed by testing for cell binding and gelatinase antagonist properties.

**Purification:** As described for $E^{375}$ —>A mutant, C-terminal domain or antibody chimeras.

5. <u>Gelatinase antagonists made as chimeras (for example antibody chimeras) by recombinant DNA methodologies in order to improve their pharmacokinetic properties.</u>

Therapeutically it may be necessary to maintain high levels of the gelatinase antagonist within the tissues for relatively long periods. This may be achieved by fusing gelatinase antagonist effector regions to another molecule such as an antibody which has properties that

improve the pharmacokinetics of the chimeric molecule. An example of this type of chimeric molecule has been reported where a receptor domain was fused to various antibody constant regions. Improved *in vivo* plasma half-lives were reported for these molecules. (Capon *et al.*, Nature 337: 525-531 (1989).

In the present examples the chimeric molecule is made by recombinant DNA technology and purified by the methods described above or by standard methodologies, but it should be clear that a man skilled in the art, would be able to make a variety of chimeric molecules with a view to improving the pharmacokinetic properties of the gelatinase antagonist, by similar means.

The cDNA encoding the human gelatinase-A C-terminal domain (for example amino acids 415-631) is fused to the 5' end of a human IgG or IgM heavy chain constant region cDNA adjacent to the $CH_1$ encoding domain. To facilitate expression and secretion of the chimeric molecule the coding sequence is attached at its 5' end to an appropriate secretion signal sequence with an initiating methionine encoding codon downstream from a promoter in a suitable expression vector. In such chimeras, gelatinase-A C-terminal domains that extend further upstream from amino-acid residue 415 can also be made in order to introduce spacing between the secretion signal sequence and the gelatinase-A domain comprised of residues 415-631.

Variants of the chimera described above but which lack the antibody hinge region are similarly constructed by fusing the gelatinase-A C-terminal domain onto the antibody constant region adjacent to the $CH_2$ domain. In the examples described above the antibody framework 4 sequence may be used as a linker between the

gelatinase-A C-terminal domain and the antibody constant region. In the latter case it may be necessary to incorporate a linking sequence such as the sequence $(Gly\ Gly\ Gly\ Gly\ Ser)_3$ between the two parts of the chimera.

Expression of chimeric molecules such as those described above may give rise to dimers or monomers. Tetrameric forms of the chimeras are similarly made by fusing the gelatinase-A C-terminal domain onto the human immunoglobulin light chain constant region and coexpressing the molecule with the hinge containing heavy chain chimera. Similar results may be obtained by co-expressing the heavy chain chimeras with non-modified light chains.

In an alternative series of chimeras the gelatinase-A C-terminal domain is fused to the C-terminus of various antibody fragments. As a linker between the two halves of the chimera the amino acid sequence referred to above may be employed or a sequence comprised of the region adjacent to the C-terminal domain of gelatinase-A, referred to above as a spacer, may also be used. The antibody partner may be the variable domain of an antibody light chain such as Rei or a human germ line variable region or may be an antibody heavy chain with fusion of the gelatinase-A C-terminal domain occurring downstream of the hinge. The variable regions can be chosen to have a particular specificity: for example for tumour antigens in order to deliver the chimeric molecule to tumour cells or be specific for gelatinase or other enzymes or cell or matrix antigens, and the linker can be designed to be cleavable in order to release the gelatinase antagonising moiety once it has been delivered to the chimeric antibody binding site. Expression of the light chain chimeras may give rise to

light chain dimers similar to Bence-Jones proteins whilst expression of the heavy chain chimeras in the presence of light chains may given rise to tetrameric chimera

In the preceding examples the effector region of the gelatinase has been taken to be the gelatinase-A C-terminal domain, or a portion of it. Alternatively chimera can be made that employ the gelatinase-A or B matrix binding domain, or portions of it, instead of the gelatinase-A C-terminal domain.

In a third method of combining gelatinase antagonist effector function with antibody fragments the effector region is used to replace one or more of the CDR's of an antibody. In this case the effector region should be comprised of a peptide derived for example from the gelatinase-A C-terminal domain or the gelatinase -A or B matrix binding domain. Such peptides can originate from epitopes in the antagonist identified by alanine scanning or random selection of continuous epitopes in the protein sequence of the said antagonist. Antibodies in which one or more CDR's have been substituted by a peptide sequence are screened for those that possess gelatinase antagonist properties such as competitive binding to cells or matrix (Murphy *et al* (1994) J. Biol. Chem. 269 (9) 6632-6636) and blocking properties in the lung colonisation assay.

**Plasmids and Cell Lines**

The Gelatinase-A expressing C127 cell line .02/3 was established using the vector pEE6hCMV.ne (Bebbington, C R. (1991). Methods: A companion to Methods in Enzymology 2, 136-145), except that the hCMV promoter was replaced by the mMT-1 promoter previously used to express stromelysin (Docherty, A J P, and Murphy, G (1990) Ann. Rheum Dis 49, 469-479). The C127 cell line expressing the gelatinase antagonist

(active site mutants ($E_{375}>Q_1$ or $E_{375}>Q_2$ and $E_{375}>A$) were made using the pEE6hCMV.ne vector.

Using $^{14}C$ labelled casein or gelatin as substrate no endogenous murine metalloproteinase activity could be detected in serum free C127 cell conditioned media. This was confirmed by examining conditioned media on casein and gelatin zymograms (Murphy G *et al.*, (1992) Biochem. J. 283, 637-641). Using similar substrates and human gelatinase-A or mixtures of human gelatinase-A and B, collagenase and stromelysin as the enzyme 0.31pg/cell day murine TIMP.2 was shown to be secreted. No murine TIMP.1 could be detected by reverse zymography Murphy *et al* (1992) J. loc. cit). These estimates assume that the murine TIMPs have specific activities that are similar to their human counterparts. The specific production rates (SPRs) of the gelatinase-A secreting transfectant (.02/3) grown for 24h in serum free DMEM was determined by ELISA (Zucker *et al* (1992) J. Immunol. Methods 148, 189-198), and was: 0.88pg/cell/24h respectively.

**Matrigel Experiments**

Non-coated transwells from Costar UK Ltd., or transwells coated with 30µg matrigel were seeded with 5 x $10^5$ C127 cells in serum free DMEM. The lower chamber contained DMEM with 10% foetal calf serum. Cells were seeded alone or in the presence of 25µg gelatinase antagonist $E_{375}>Q$. The antagonist was added to the upper chamber 30 minutes before addition of the cells. The total number of cells that traversed the filter was counted after incubation at 37°C for 72h, essentially as previously described except that the total number of cells passing through the filter were counted rather than being quantified by labelling (Repesh, L A (1989) Invasion Metastasis 9, 192-208). The levels of significance (p) were calculated by two tailed t-test analysis using the StatView programme on an Apple Macintosh. Each bar in the figures represents the mean of 3 replicates and the error bars are +/- one standard deviation.

**Lung Colonisation Assays**

Groups of 10 female Balb/c nu.nu mice were injected into the tail vein with 1x$10^6$ C127 (.02/3) transfectants. Lung nodules were scored at 4 weeks

after staining in Bouins fixative (Fidler, I J (1978) Methods Cancer Res. 15, 399-439).

Experiments employing the gelatinase antagonist active site $E_{373}$>A were undertaken as described above except the cells were preincubated in the presence of the dead Gelatinase A enzyme (mutation E to A) prepared as described in Examples 1 and 2 and in Crabbe *et al*; (Biochemistry (1994) 33, 6684-6690), for 1 hour. The cells were incubated at 37°C at a concentration of 25µg dead enzyme/5 x $10^5$ cells in DMEM. The cells were washed twice, resuspended in DMEM and placed on ice prior to injection.

As a control C127.02/3 cells were incubated at 37°C for 1 hour in DMEM without dead enzyme before being washed and resuspended as above.

For co-injection of 25µg of dead enzyme was added per 5 x $10^5$ cells immediately prior to injection.

In all cases the cells were injected into balb/c nu.nu mice at a dose of 5 x $10^5$ cells per mouse in a volume of 200µl DMEM.

4 weeks following injection the mice were sacrificed by $CO_2$ asphyxiation, the lungs removed, washed in PBS and fixed in 2% formalin for one hour followed by overnight fixation in Bouins stain. The lungs were scored for nodules the following day by examination under light microscopy. The results are shown in Figure 8.

Error bars represent +/- one standard deviation. All experiments were conducted in compliance with home office regulations and according to UK CCCR guidelines for the welfare of animals with experimental neoplasia.

**EXAMPLE 5**

**Assays of Cell Invasion/Migration**

1. Matrigel

As described above.

2. Metastatic Cell Spread

As already described. Also, the ability to inhibit the metastatic spread of A375 cells to the bone could be studied (refs. F. Arguello, R B Baggs & C N Frantz (1988) Cancer Res. 48, 6876-6881; M Nakai, G R Mundy, P J Williams, B Boyce and T Yoneda 1992) Cancer Res. 52, 5395-5399).

3. Experimental Allergic Encephalomyelitis (EAE)

(Martenson, R E (1984). In Experimental Allergic Encephalomyelitis; A Useful Model for Multiple Sclerosis, ed E C. Alvord, Jr., pp 146-511. New York: Liss). Look at the invasion of T-cells. Represents a model of multiple sclerosis but also other autoimmune disorders or T-cell mediated inflammation. Cell-bound gelatinase A might be involved in movement of T-cells. (A M Romnanic, J A Madri (1994) J. Cell Biol. 125, 1165-1178).

4. Experimental Arthritis

For example the gelatinase antagonist is evaluated in a zymosan induced arthritis in the mouse as previously described by Follard and Terlain (1988) (Agents and Actions 25 1/2) or in the Dumonde and Glynn rabbit model ((1962)B.J. Exp. Pathol. 43, 373-383) as for example described by Lewthwaite *et al* (1994) (The Journal of Rheumatology 21 3, 467-472).

5. Experimental Atherosclerosis

There is some evidence that matrix metalloproteinase are involved in aortic smooth muscle proliferation (Southgate *et al* (1992) Biochem J. 288 93-99). The gelatinase antagonist is therefore evaluated in for example a model of restinosis following balloon angioplasty as previously described (Bennett *et al* (1994) J. Clin. Invest. 93 820-828).

6. Experimental Ulcerative Colitis and Crohn's Disease

There is some evidence that matrix integrity is lost during inflammatory cell infiltration in *ex vivo* models of inflammatory bowel disease (Lionetti *et al*, (1993) Gastroenterology 105, 373-381) and

*in vivo* when biopsies of human material are examined (Murch *et al.*, (1993) The Lancet 341, 711). The gelatinase antagonist is evaluated for its beneficial effect in the *ex vivo* model as previously described (Lionetti *et al*, (1993) J. loc. cit.).

5

**CLAIMS**

1. A method of treating diseases in which gelatinase mediated cell migration and invasion is an essential feature of the pathology of the disease comprising administering an effective amount of an antagonist which inhibits said gelatinase mediated cell migration and invasion.

2. A method of treating cancer according to Claim 1 by inhibiting the invasion of tumour cells comprising administering an effective amount of a gelatinase antagonist which inhibits gelatinase mediated cell migration and invasion.

3. A gelatinase antagonist which has altered catalytic activity with respect to wild type gelatinase and which inhibits gelatinase mediated cell migration and invasion with the proviso that said antagonist is not the isolated natural C-terminal region of gelatinase A.

4. A gelatinase antagonist according to Claim 3 in which the glutamic acid residue at position 375 has been deleted or modified or replaced by another amino acid or derivative thereof.

5. A gelatinase antagonist according to Claim 4 wherein said antagonist is a full length gelatinase derivative in which the glutamic acid residue at position 375 is replaced by a glutamine, alanine or aspartic acid residue.

6. A therapeutic, pharmaceutical, imaging or diagnostic composition comprising a gelatinase antagonist which inhibits the binding of gelatinase to target cells and/or extracellular matrix optionally labelled or coupled to an antibody or drug molecule and a pharmaceutically acceptable excipient.

7. A method of delivering a drug or antibody to a target site characterised in that said drug or antibody is directly or indirectly linked to a gelatinase antagonist said antagonist inhibiting gelatinase mediated cell migration and invasion.

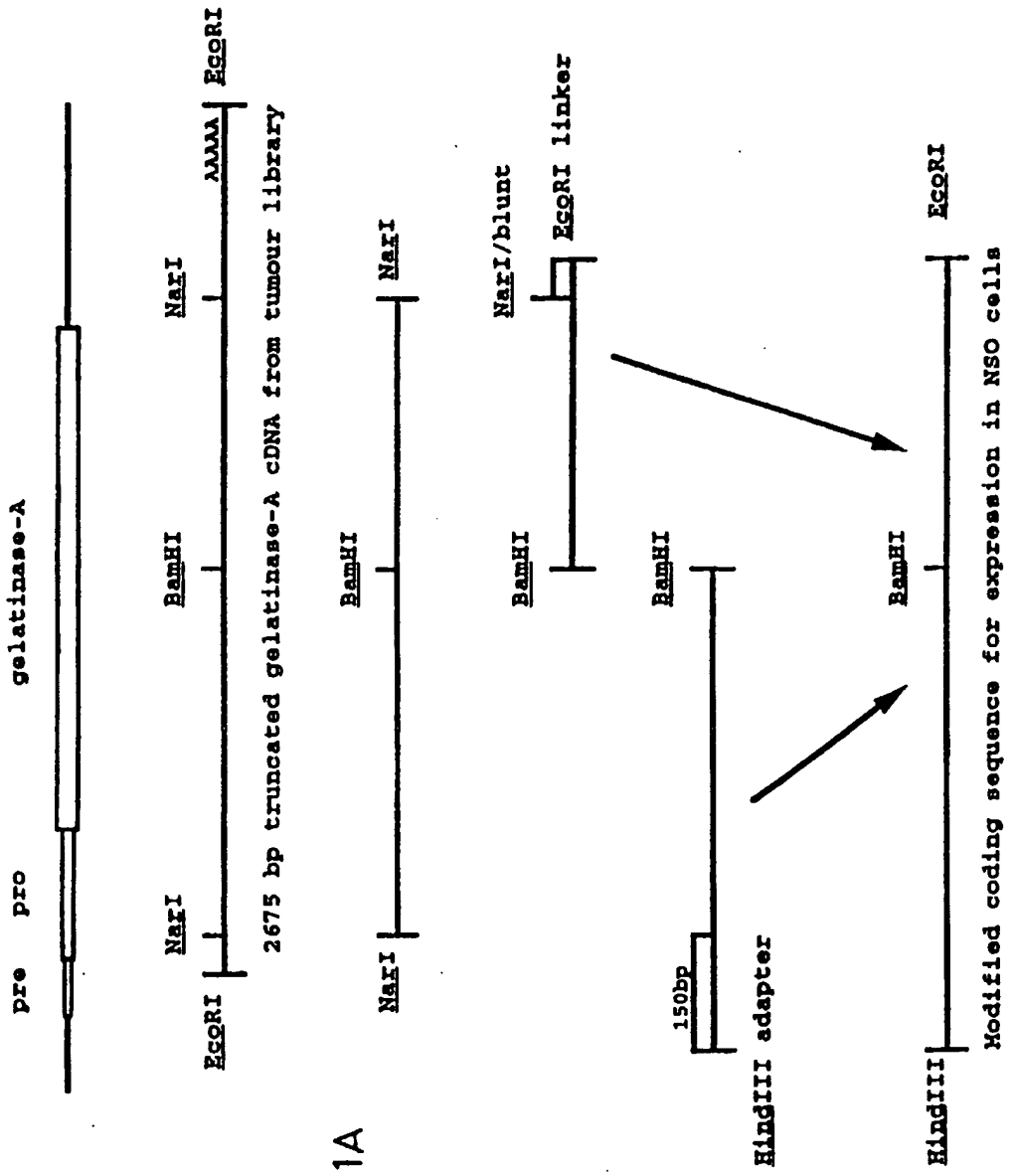

pre pro gelatinase-A
NarI
EcoRI
2675 bp truncated gelatinase-A cDNA from tumour library
BamHI
NarI
AAAA
EcoRI
BamHI
NarI
NarI/blunt
EcoRI linker
BamHI
150bp
HindIII adapter
BamHI
EcoRI
HindIII
Modified coding sequence for expression in NSO cells

FIG.1A

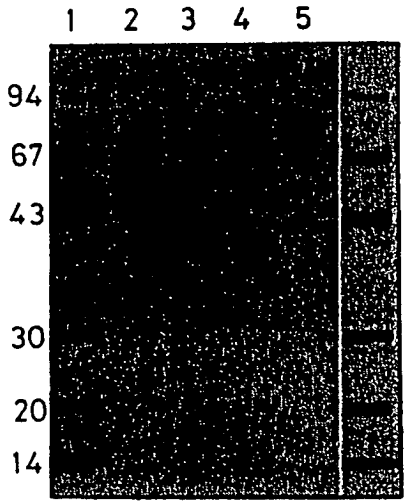
HindIII
AGCTTGCCACCATGGAGGCTCTAATGGCTAGAGGCGCTCTCACCGGTCCTCTGAGAGCTCTCTGTCTCCTGGGC
ACGGTGGTACCTCCGAGATTACCGATCTCCGCGAGAGTGGCCAGGAGACTCTCGAGAGACAGAGGACCCG
M E A L M A R G A L T G P L R A L C L L G

NarI
TGCCTGCTGAGCCACGCTGCTGCTGCTCCTTCTCCCATCATCAAGTTCCCTGGCGATGT CGCCCCCAAA..
ACGGACGACTCGGTGCGACGACGACGAGGAAGAGGGTAGTAGTTCAAGGGACCGCTACAGC GGGGGTTT..
C L L S H A A A A P S P I I K F P G D V A P K
1 5 10 15

FIG.1B

SUBSTITUTE SHEET (RULE 26)

FIG.3



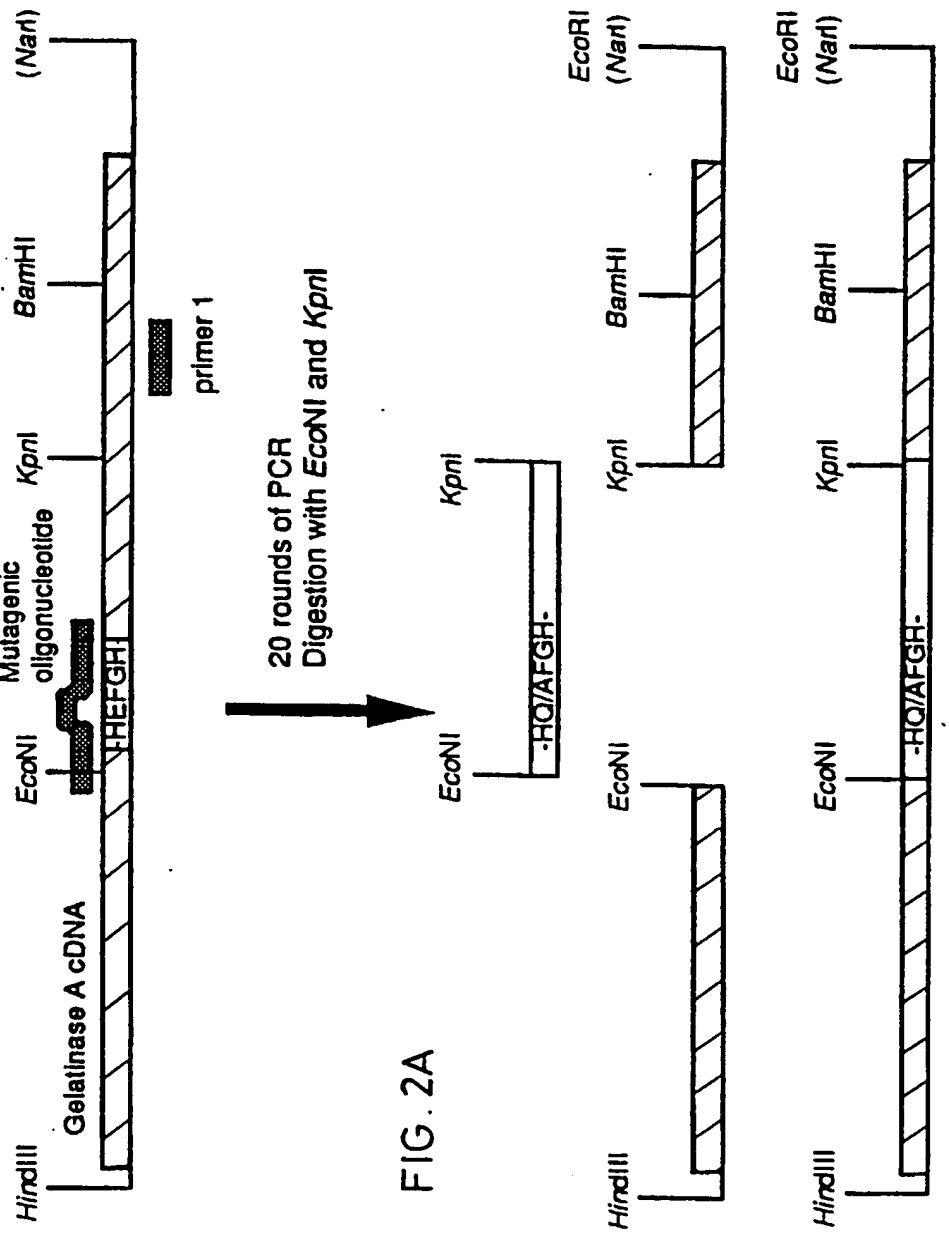

EcoRI (NarI)
BamHI
KpnI
Mutagenic oligonucleotide
EcoNI
-HEFGH-
primer 1
Gelatinase A cDNA
HindIII
20 rounds of PCR
Digestion with EcoNI and KpnI
KpnI
-HQIAFGH-
EcoNI
EcoRI (NarI)
BamHI
KpnI
EcoNI
HindIII
EcoRI (NarI)
BamHI
KpnI
EcoNI
-HQIAFGH-
HindIII

FIG. 2A

FIG.2B

360 365 370 375

G F C P D Q G G Y S L F L V A A H E F G H A

5′-GGGGCTTCTGCCCTGACCAAGGGTACAGCCTGTTCCTCGTGGCAGCCCACGAGTTTGGCCACGCC-3′

3′-CCCCGAAGACGGGACTGGTTCCCATGTCGGACAAGGAGCACCGTCGGGTGCTCAAACCGGTGCGG-5′

*EcoNI*

**Mutagenic oligonucleotide : 1** -H Q F G-

5′-GGGGCTTCTGCCCTGACCAAGGGTACAGCCTGTTCCTCGTGGCAGCCCACCAGTTTGGCC-3′

**Mutagenic oligonucleotide : 2** -H A F G-

5′-GGGGCTTCTGCCCTGACCAAGGGTACAGCCTGTTCCTCGTGGCAGCCCACGCCTTTGGCC-3′

**Mutagenic oligonucleotide : 3** -H D F-

5′-GGGGCTTCTGCCCTGACCAAGGGTACAGCCTGTTCCTCGTGGCAGCCCACGACTTTGGCC-3′

FIG. 4

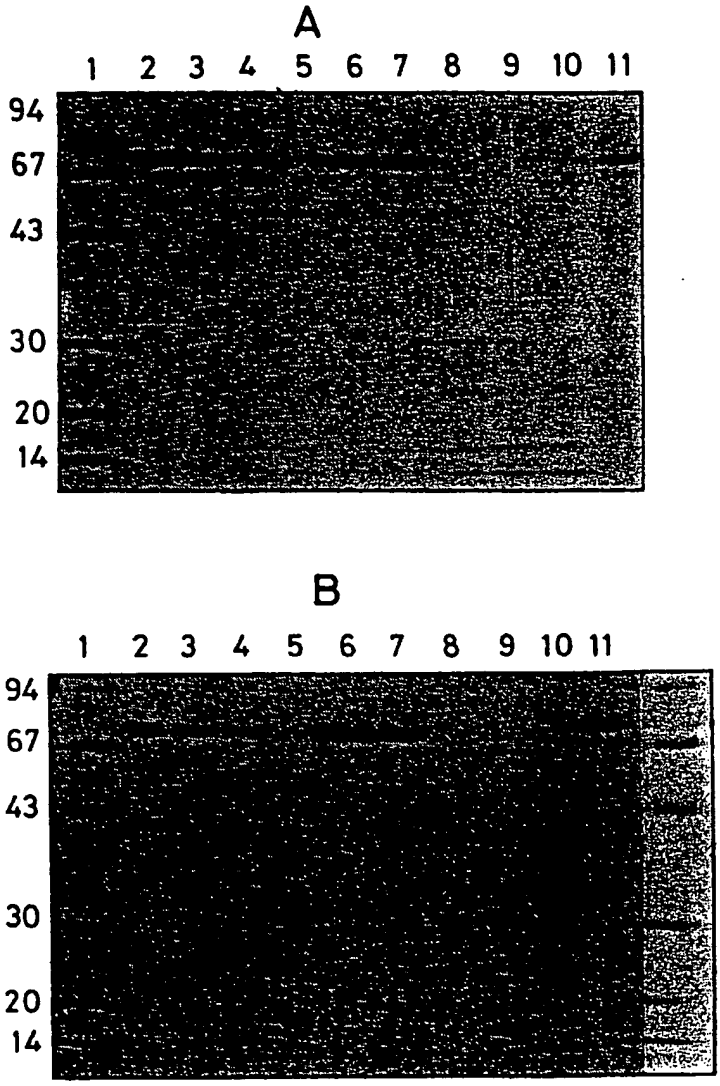

A
1 2 3 4 5 6 7 8 9 10 11
94
67
43
30
20
14
B
1 2 3 4 5 6 7 8 9 10 11
94
67
43
30
20
14

FIG.5

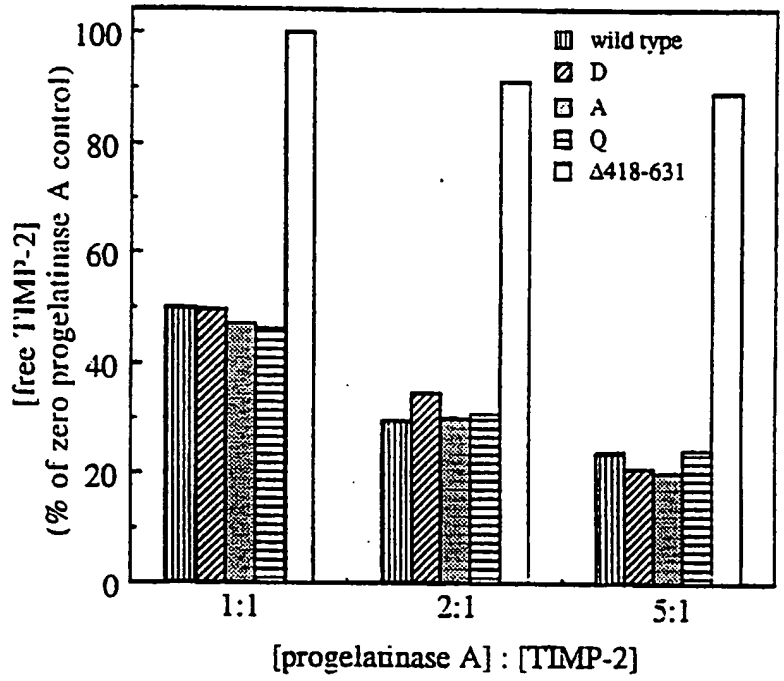
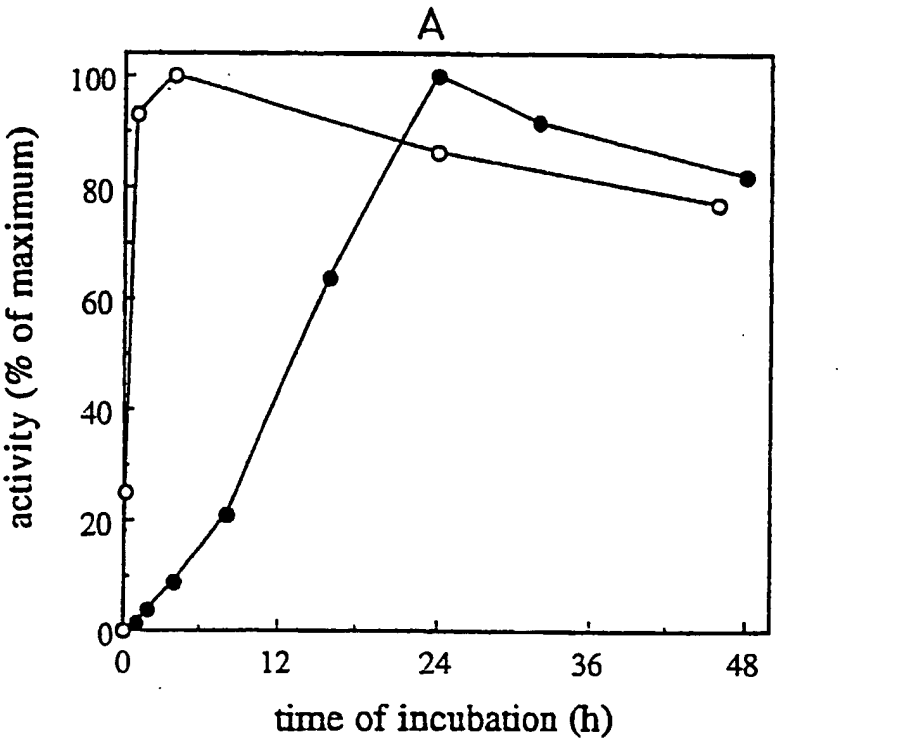
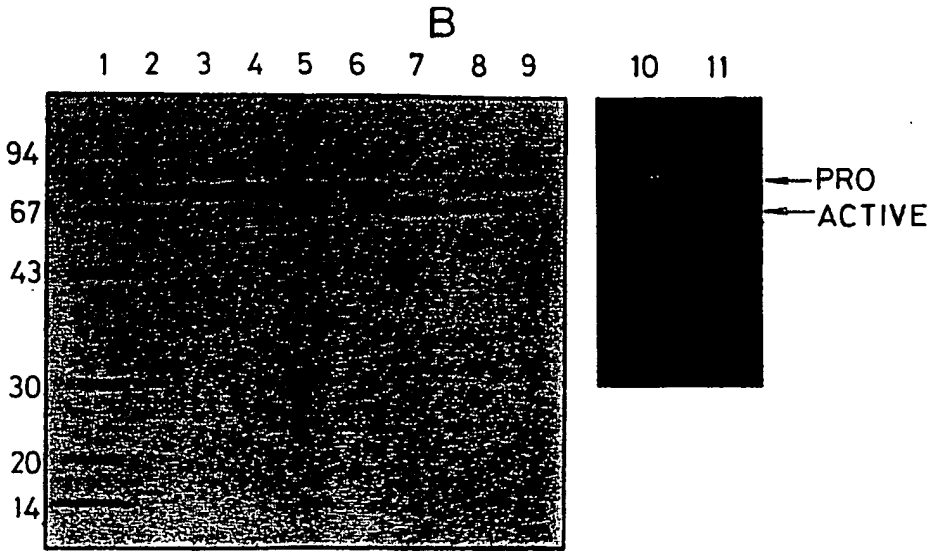

100
80
60
40
20
0
[free TIMP-2]
(% of zero progelatinase A control)
wild type
D
A
Q
Δ418-631
1:1
2:1
5:1
[progelatinase A] : [TIMP-2]

FIG. 6

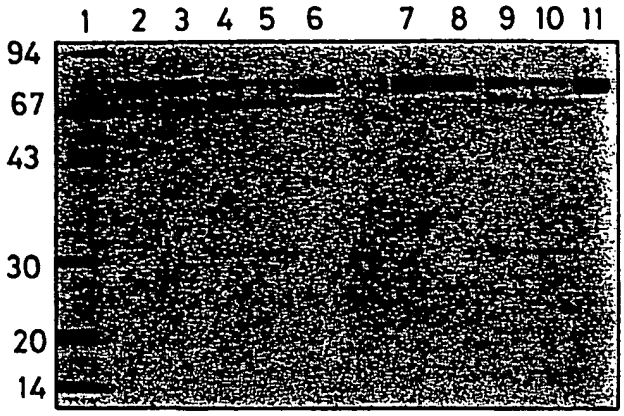

A
activity (% of maximum)
100
80
60
40
20
0
0
12
24
36
48
time of incubation (h)


B
1 2 3 4 5 6 7 8 9
10 11
94
67
43
30
20
14
PRO
ACTIVE

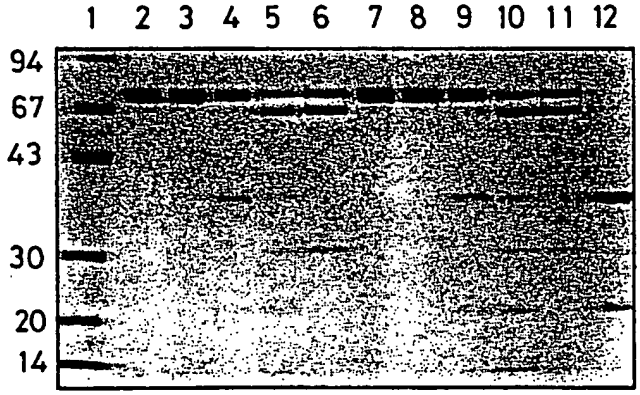
8/9

FIG. 7

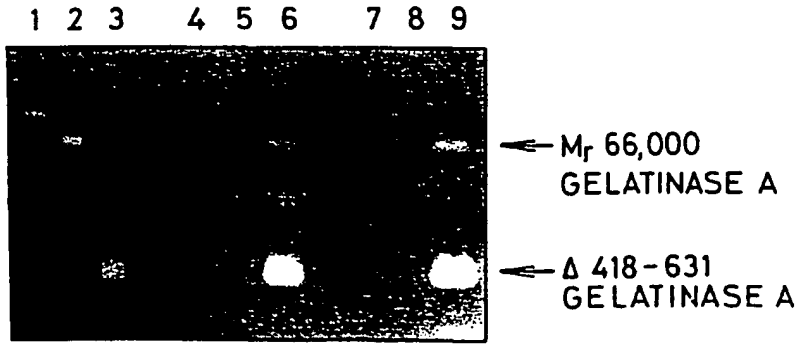
A


1 2 3 4 5 6 7 8 9 10 11
94
67
43
30
20
14

B


1 2 3 4 5 6 7 8 9 10 11 12
94
67
43
30
20
14
Δ 418-631 GELATINASE A
C-TERMINAL DOMAIN

C


1 2 3 4 5 6 7 8 9
Mr 66,000 GELATINASE A
Δ 418-631 GELATINASE A

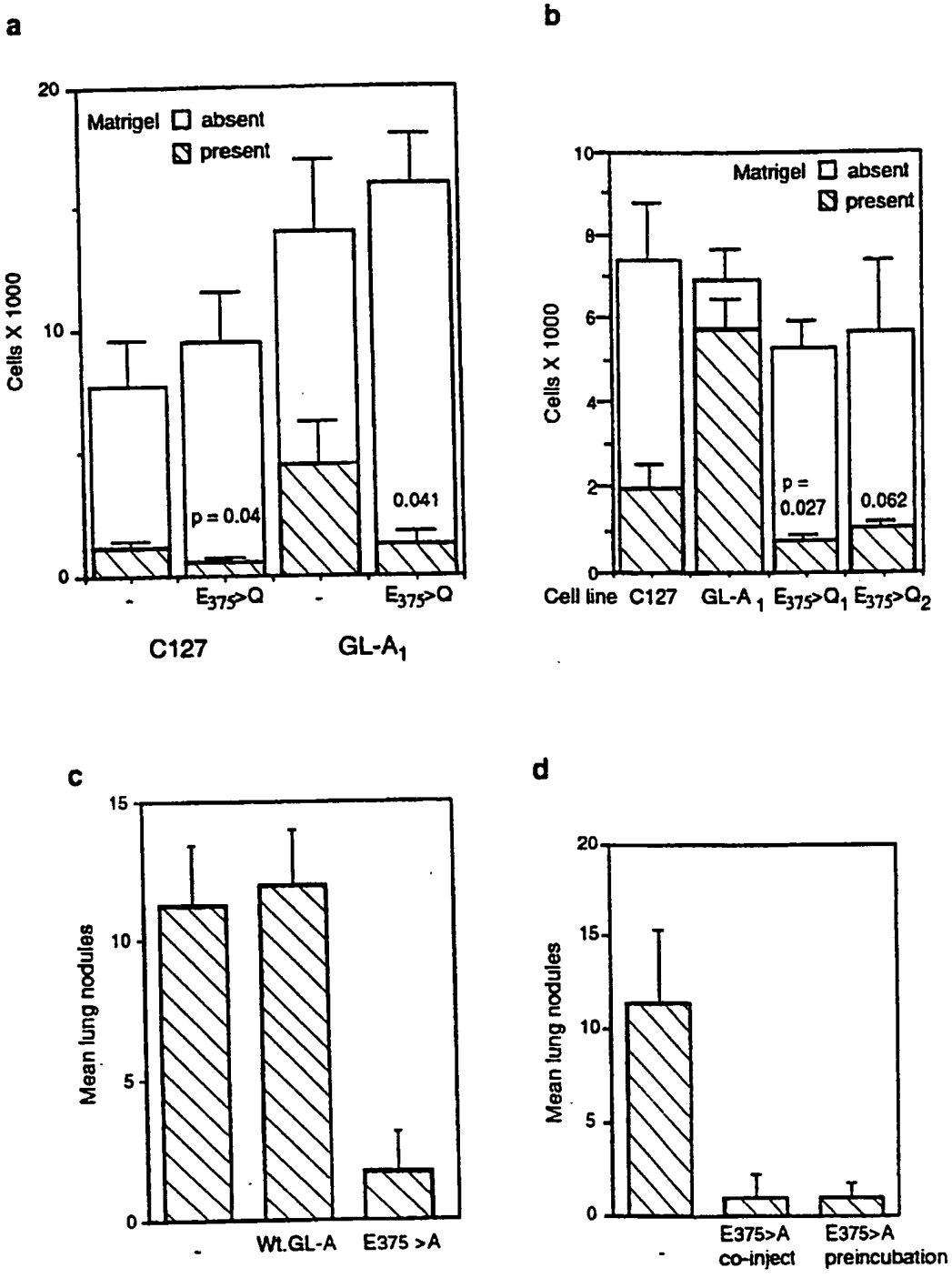

a
Matrigel absent
present
Cells X 1000
20
10
0
p = 0.04
0.041
-
E375>Q
-
E375>Q
C127
GL-A1
b
Matrigel absent
present
Cells X 1000
10
8
6
4
2
0
p = 0.027
0.062
Cell line C127
GL-A 1
E375>Q1
E375>Q2
c
Mean lung nodules
15
10
5
0
-
Wt.GL-A
E375 >A
d
Mean lung nodules
20
15
10
5
0
-
E375>A co-inject
E375>A preincubation

FIG. 8

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| 9322774.2 | 4 November 1993 (04.11.93) | GB |

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(74) Agent: MERCER, Christopher, Paul; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

(54) Title: GELATINASE ANTAGONISTS USED FOR TREATING CANCER

(57) Abstract

This invention describes a method of treating diseases in which gelatinase mediated cell migration and invasion is an essential feature of the pathology of the disease. The method comprises administering an effective amount of an antagonist which inhibits gelatinase mediated cell migration and invasion.

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# INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/GB 94/01485

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/64 A61K38/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | CANCER RESEARCH, vol.52, 15 April 1992 pages 2353 - 2356 A. MELCHIORI ET AL 'Inhibition of tumor cell invasion by a highly conserved peptide sequence from the matrix metalloproteinase enzyme prosegment' see the whole document --- | 1-3,6 |
| Y | JOURNAL OF BIOLOGICAL CHEMISTRY., vol.268, no.19, 5 July 1993, BALTIMORE US pages 14033 - 14039 A. STRONGIN ET AL 'Plasma membrane-dependent activation of the 72-kDa type IV collagenase is prevented by complex formation with TIMP-2' see page 14036, right column, paragraph 2 - page 14038, left column, paragraph 1 --- -/-- | 1-7 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 1 December 1994 | 14 -12- 1994 |

| Name and mailing address of the ISA | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016 | Van der Schaal, C |

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | TRENDS IN BIOTECHNOLOGY.,<br>vol.10, June 1992, CAMBRIDGE GB<br>pages 200 - 207<br>A. DOCHERTY ET AL 'The matrix metalloproteinases and their natural inhibitors: prospects for treating degenerative tissue diseases'<br>see page 204, right column, line 17 - line 24 | 1,2,6 |
| Y | see page 206, left column, line 24 - line 34 | 1-7 |
| Y | CANCER RESEARCH,<br>vol.52, 15 October 1992<br>pages 5845 - 5848<br>H. EMONARD ET AL 'Tumor cell surface-associated binding site for the Mr 72,000 type IV collagenase'<br>cited in the application | 1-7 |
| X | ABSTRACTS OF PAPERS AMERICAN CHEMICAL SOCIETY,<br>vol.205, no.1-2, 28 March 1993<br>page MEDI 92<br>M. COCKETT ET AL 'Tumor metastasis and gelatinase' | 1,2,6 |
| Y | see abstract | 1-7 |
| Y | EP,A,0 347 931 (AZUMA, ICHIRO) 27 December 1989<br>see the whole document | 1-6 |
| Y | GB,A,2 209 526 (WASHINGTON UNIVERSITY) 17 May 1989<br>see the whole document | 4,5 |
| Y | WO,A,86 00527 (DANA-FARBER CANCER INSTITUTE) 30 January 1986<br>see abstract | 7 |
| A | BIOCHEMICAL JOURNAL,<br>vol.283, 1992<br>pages 637 - 641<br>G. MURPHY ET AL 'The C-terminal domain of 72 kDa gelatinase A is not required for catalysis, but is essential for membrane activation and modulates interactions with tissue inhibitors of metalloproteinases'<br>see abstract | 1-3,6,7 |

-/--

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | CANCER RESEARCH,<br>vol.53, 1 July 1993<br>pages 3159 - 3164<br>W. MONSKY ET AL 'Binding and localization of Mr 72,000 matrix metalloproteinase at cell surface invadopodia' | 1-3,6,7 |
| P,Y | BIOCHEMICAL SOCIETY TRANSACTIONS,<br>vol.22, no.1, February 1994<br>pages 55 - 57<br>M. COCKETT ET AL 'Metalloproteinase domain structure, cellular invasion and metastasis'<br>see the whole document | 1-7 |
| P,Y | BIOCHEMISTRY,<br>vol.33, 31 May 1994<br>pages 6684 - 6690<br>T. CRABBE ET AL 'Mutation of the active site glutamic acid of human gelatinase A: effects on latency, catalysis and binding of tissue inhibitor of metalloproteinases-1'<br>see the whole document | 4,5 |
| P,A | JOURNAL OF BIOLOGICAL CHEMISTRY.,<br>vol.269, no.9, 4 March 1994, BALTIMORE US<br>pages 6632 - 6636<br>G. MURPHY ET AL 'Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant'<br>see the whole document | |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB94/01485

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 1,2 and 7 are (partially) directed to a method of treatment or a diagnostic method practised on the human/animal body the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna ıl Application No
PCT/GB 94/01485

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